Medical and Pediatric Oncology 27:484–489 (1996)

Multiple Genetic Abnormalities of 11p15 in Wilms' Tumor

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Wilms' tumor has served as a model of multiple genetic alterations in childhood cancer. This review summarizes work in our laboratory identifying several of these alterations. These include the localization to 11p15 of an embryonal tumor suppressor gene and at least one gene for Beckwith-Wiedemann syndrome, which predisposes to Wilms' tumor; as well as a novel mutational mechanism in man, loss of imprinting.

INTRODUCTION

Wilms' Tumor (WT) was originally believed to represent a relatively simple single gene model of carcinogenesis [1]. It was one of the original paradigms of Knudson and Strong's two-hit hypothesis of carcinogenesis, representing the inactivation of the two copies of a tumor suppressor gene. Patients with germline mutations (first hit of a Knudsonian gene) develop bilateral tumors at an earlier age than patients with unilateral tumors, who must develop both hits (inactivation of both alleles of the gene) in somatic cells. Knudson's model predicts: (1) frequent mutations of the gene in sporadically arising tumors; (2) loss of heterozygosity (LOH) involving the gene in tumors; (3) suppression of tumorigenicity by reintroduction of the gene into tumor cells lacking a normal copy; and (4) familial cases involving germline mutations of the gene. Experiments addressing each of these predictions suggest that WT involves multiple genes on chromosomal band 11p15, as well as a novel genetic mechanism, loss of imprinting.

A Tumor Suppressor Gene on 11p15: Isolation Within Subchromosomal Transferable Fragments

Fearon, Vogelstein, and I showed several years ago that loss of heterozygosity (LOH) of polymorphic markers on chromosomal arm 11p is a common feature of WT [2]. *WT1*, the WT gene isolated from 11p13 by two laboratories, was found to be a transcription factor with zinc finger DNA binding motifs [3,4]. Even though the function of *WT1* is not entirely clear, experiments involving *WT1* knockout mice suggest that *WT1* is necessary for normal kidney development [5]. It shares a DNA binding motif with EGR1 [6], but it may also serve as a transcriptional inhibitor of other genes [7]. *WT1* does follow Knudson's two-hit model for WAGR-associated WT [8] in some sporadic tumors [9]. However, *WT1* appears to account for only a small fraction (10%) of 11p-related WT [10,11], and *WT1* mutations are not a general feature of embryonal tumors.

We and others have shown that an 11p15 gene or genes appear to be involved much more commonly than *WT1* in Wilms' tumors and more generally in other embryonal malignancies. Most LOH on 11p does not specifically involve 11p13 (which includes *WT1*), but the common region of overlap of LOH is 11p15 [12]. LOH of 11p15 has subsequently been observed in other embryonal tumors as well, including rhabdomyosarcoma and hepatoblastoma [13]. Frequent loss of 11p15 has also been found in many common cancers, including bladder [14], ovary [15], breast [16], and lung [17]. However, it cannot be assumed that embryonal tumor suppressor genes are also involved in common malignancies, as the LOH data suggest differing regions of involvement [16,18,19].

The second indication of the importance of 11p15 is that this band, rather than 11p13, suppresses tumorigenicity of G401 cells, which are derived from either a Wilms' or rhabdoid tumor of the kidney [20]. Thus, while an intact chromosome 11 suppresses tumorigenicity, a chromosome 11 lacking a very large region including 11p14 and 11p15, but containing 11p13, does not, while a chromosome 11 lacking 11p13 still suppresses growth [21].

In order to prove the existence of an 11p15 tumor suppressor gene and to localize it precisely, we developed a novel direct genetic complementation strategy using subchromosomal fragments of several megabases to bridge the gap between whole chromosomes and yeast artificial chromosomes (YACs) in genetic complementation experiments [22]. In this approach, we transfect a selectable mammalian gene into mouse cells containing a single independently selectable human chromosome. We transfer chromosomes by microcell fusion and double

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Received August 6, 1995; accepted February 6, 1996.

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select for the human chromosome and the marker gene. We then perform microcell fusion in order to isolate individual marker-containing chromosomal subfragments [22]. Unlike conventional radiation hybrids [23], these subchromosomal transferable fragments (STFs) can be transferred individually to any mammalian cell of choice, by virtue of the selectable marker gene within them. They can therefore be used to isolate growth inhibitory genes, such as tumor suppressor genes.

We constructed a library of 85 STFs from chromosome 11. We showed that these STFs were stably transferable to cells, by transferring them from their host mouse cells to Chinese hamster ovary cells, and performing pulse field gel electrophoresis (PFGE) of the DNA from donor and recipient cells. Hybridization with a human repetitive sequence allows visualization of the individual human PFGE fragments. These were essentially identical between donor and recipient cells, and the neo gene always mapped to Alu-positive human PFGE fragments, confirming that the selectable marker was integrated within the human DNA [22].

We then transferred ten 11p15 and two 11q STFs to the rhabdomyosarcoma cell line RD, because RD had been previously shown to undergo in vitro growth arrest after introduction of an entire chromosome 11 [24], making it easy to score for growth suppressor activity. Beckwith-Wiedemann Syndrome (BWS) patients are also at risk for rhabdomyosarcoma, which also shows LOH of 11p15. When some of the STFs were introduced into RD cells, they appeared to senesce in vitro, showing marked growth reduction and striking morphological changes. Using SSCP-PCR to detect polymorphisms distinguishing the donor from the recipient cell DNA, we were able to confirm that donor DNA was present within the senescing colonies [22].

Furthermore, we could perform precise mapping of the STFs and identify the region containing the suppressor activity. There was no relationship between growth inhibition and the presence of 11p13 or *WT1,* nor was *WT1* expressed in tumor-suppressing STFs. However, there was a strong correlation of growth suppression with a 3 Mb region (more recently narrowed to 2 Mb) of 11p15 [22]. We localized this activity to a region between the β-globin gene cluster and the *IGF2* gene.

These experiments are important for three reasons. First, they establish unequivocally the existence of at least one tumor suppressor gene on 11p15. Second, they provide a general strategy for isolating tumor suppressor genes which are not amenable to conventional expression cloning, because cells that contain them are selected against. This strategy should prove generally useful to laboratories trying to establish the critical regions involved that are indicated only indirectly by large chromosomal losses reflected by LOH. This can also be useful for cloning large genes not amenable to other approaches. Third, these studies have served as a molecular guidepost to our own and other laboratories that have attempted to identify tumor suppressor genes on 11p15.

A Beckwith-Wiedemann Syndrome Gene on 11p15

The most common familial cause of WT is Beckwith-Wiedemann syndrome (BWS), affecting 1 in 10,000 children. BWS is a disorder of abnormally increased growth, characterized by enlarged kidneys and other organs, hemihypertrophy, macroglossia, neonatal hypoglycemia, and craniofacial and ear anomalies [25,26]. BWS patients are at increased risk (approximately 20%) of several embryonal malignancies (WT, rhabdomyosarcoma, hepatoblastoma, and gonadoblastoma), as well as adrenocortical carcinoma [27]. Most cases of BWS occur sporadically, although some families show dominant inheritance with incomplete penetrance [28], and apparently discordant monozygotic twins have been reported [29]. Rare germline karyotypic abnormalities involve both balanced and unbalanced rearrangements of 11p. The balanced rearrangements specifically involve 11p15 and always the maternaly derived chromosome. In contrast, the unbalanced rearrangement breakpoints may occur anywhere within 11p but always include 11p15, and always the paternal chromosome is duplicated (reviewed in [30,31]). At the time we began studying BWS, it was unclear where the BWS gene resides, because of the confusing chromosomal evidence (and the rarity of chromosomal alterations) and the apparent paradox that, at the time, the only known Wilms' tumor gene mapped to 11p13.

Because of the uncertainty regarding localization of the BWS gene, we performed genetic linkage analysis in two large kindreds. We found strong evidence for tight linkage between BWS and the insulin locus on 11p15 ($z = 3.60, \Phi = 0.00$), with no evidence for recombination between the two loci [32]. Conversely, BWS was unlinked to markers on 11p13, which harbors the *WT1* gene [32]. The additive lod score for linkage to 11p15 from our data and that of another laboratory [33] was 6.2 with no recombinants. This is consistent with localization to 11p15.4 or 11p15.5. Thus, it appeared that a general embryonal tumor suppressor gene on 11p15, not limited in its target to WT, might also be involved in BWS. Given the relatively small number of informative meioses in our families and in the literature, and the incomplete penetrance of the BWS gene, we realized early that physical cloning efforts rather than genetic linkage analysis would be necessary to further localize the gene. We thus focused on balanced rearrangement breakpoints which specifically involve 11p15.

We derived STSs from probes near each breakpoint in order to identify YACs that contain them. We and our colleagues (J. Hoovers, M. Mannens, University of Amsterdam) then mapped each of these YACs by FISH to

each of the BWS cells lines harboring balanced germline chromosomal rearrangements. YACs spanning seven breakpoints were identified in this manner. Five BWS breakpoints were found to be clustered near but centromeric to *IGF2* and *H19.* Hybridization with known probes, and with end clones derived from YACs by PCR amplification, indicated that five YACs spanning these five breakpoints, and excluding *IGF2* and *H19,* formed a 700-kb overlapping YAC contig, representing a maximum distance among them of 700 kb at this level of resolution [34].

However, three additional breakpoints were located 4.0–5.2 Mb centromeric to this cluster, suggesting that the BWS breakpoints represent more than one gene [34]. A complete cosmid contig through this region has been generated, and candidate genes within it are being isolated. The sizes of the cosmids establish a maximum physical distance of only 295–320 kb spanning the cluster of 5 BWS balanced rearrangement breakpoints. Thus, this cluster is of a size that could reasonably be expected to harbor a gene [34]. We also molecularly cloned a balanced translocation breakpoint, t(11p15.5; 22q11), from a malignant rhabdoid tumor. We have found that this rhabdoid tumor breakpoint falls within the BWS breakpoint cluster, suggesting that the same gene may be involved in both [34]. An important advantage of our two independent approaches, positional cloning of BWS chromosomal rearrangement breakpoints [34] and genetic complementation using STFs [22], is that we were able to determine that the BWS breakpoints in this cluster corresponded to the tumor suppressor region defined by STFs [34].

Genomic Imprinting of 11p15 Genes and Loss of Imprinting in Wilms' Tumor

Genomic imprinting is a differential modification of the two parental chromosomes in the gamete or zygote, leading to differential function of the two chromosomes in the offspring. Imprinting is by definition reversible, and it usually refers to monoallelic expression of a specific parental allele of a gene.

Genomic imprinting has been thought for twenty years to be important in human neoplasia, because androgenetic embryos (46 chromosomes all derived from the father) give rise to hydatidiform moles [35], and parthenogenetic embryos (46 chromosomes, all derived from the mother) give rise to complete ovarian teratomas [36]. A role for imprinting of 11p15 genes in embryonal tumors is suggested by the fact that LOH in WT and other embryonal tumors almost always involves loss of the maternal allele [37]. This observation led Sapienza and colleagues to propose a model in which the first hit inactivating a Knudsonian embryonal tumor suppressor gene on 11p15 can be aberrant imprinting of the paternal allele, rather than mutation, followed by loss of the maternal allele [38]. Preferential LOH of a specific parental allele has been described in several other tumor types (reviewed in [39]).

An alternative model for the role of genomic imprinting in carcinogenesis derives from our discovery, described below, of loss of imprinting (LOI) of the insulin-like growth factor-II gene (IGF2), an important autocrine growth factor in many tumors, including WT, rhabdomyosarcoma, and hepatoblastoma. Thus, LOI would lead to activation of the normally silent maternal allele of a growth-promoting gene.

BWS may also involve imprinted genes, because of the difference in parental origin of balanced rearrangements (maternal) and unbalanced duplications (paternal) [30,31]. Furthermore, 10% of patients show paternal uniparental disomy (UPD), in which several megabases of the maternal copy of 11p15 are replaced with a duplicated paternal copy [40]. UPD could involve either pathogenic model of imprinting, i.e., loss of a gene normally expressed only from the maternal allele, or duplication of a gene normally expressed only from the paternal allele.

We suspected that some genes on 11p15 might be imprinted for two reasons. First, Junien and colleagues had observed paternal uniparental disomy of 11p15 in about 10% of BWS patients [40,41]. Second, the human homologues of two genes known to be imprinted in mouse, insulin-like growth factor-II (IGF2) [42] and *H19* [43], are both syntenic to 11p15 in humans. *IGF2* is an important autocrine growth factor in many tumor types, including WT and rhabdomyosarcoma [44–46]. *H19* is an untranslated RNA of less clear function [47], which was reported to cause embryonic lethality when expressed as a transgene [48], and to suppress the growth of tumor cells when overexpressed after transfection [49]. However, *H19* does not lie within the region of tumor suppressor activity we identified using STFs [22].

We thus sought to determine whether the *IGF2* and *H19* genes are imprinted in man, and, if so, whether they are altered in WT. At the time, no human gene had been shown at the molecular level to be imprinted. Using transcribed polymorphisms in both *IGF2* and *H19,* including a common but previously inapparent subtle variation in the length of a long dinucleotide repeat in *IGF2,* we found that both of these genes show monoallelic expression in human tissues. As in the mouse, we found that the human *IGF2* gene was expressed exclusively from the paternal allele and *H19* exclusively from the maternal allele [50].

To assess imprinting in cancers, 42 WTs were screened for those not exhibiting LOH of 11p15. Surprisingly, of 16 informative tumors retaining heterozygosity, 11 (69%) expressed both maternal and paternal alleles of *IGF2, H19,* or both. The frequency of biallelic expression was greater for *IGF2* (77%) than for *H19* (29%) [50]. We also found LOI in rhabdoid tumors [50], and a second laboratory also identified LOI of *IGF2* in WT [51]. This

work represents the first evidence for normal imprinting of specific human genes, and loss of imprinting in cancer, a novel mutational mechanism.

We and others have recently found that LOI also involves rhabdomyosarcoma and hepatoblastoma [52,53], tumors for which BWS patients are also at risk. Furthermore, *IGF2* shows LOI in normal tissues of some BWS patients [54,55]. However, we do not believe that *IGF2* is the only gene involved in BWS, for two reasons. First, it undergoes LOI in only 10% of BWS patients [54,55]. Second, we have identified rearranged transcripts in BWS chromosomal breakpoint patients, and *IGF2* is not involved in these rearrangements, nor do the rearrangements appear to activate the maternal allele of *IGF2* [34]. Nevertheless, it is striking that all of the balanced BWS rearrangement breakpoints are of maternal origin [30,31]. Thus, one or more genes in the BWS/tumor suppressor coincident region may be imprinted.

An interesting gene in the BWS/tumor suppressor coincident region is p57^{KIP2}, a cyclin-dependent kinase inhibitor [34], previously mapped to 11p15 [56]. Overexpression of p57 causes growth arrest of mouse p53$^-$Rb$^-$ SAOS-2 cells [56]. p57 is homologous in its inhibitory domain to p21/waf1, a cdk inhibitor that is a potential mediator of p53 tumor suppression [57]. p21 also serves as a checkpoint for G1 arrest in proliferating cells [58] and may contribute to cell cycle arrest in terminally differentiating cells [59]. p57 is also homologous in its inhibitory domain to p16, which is mutated in many tumor cell lines [60] as well as some primary tumors [61]. We are currently determining whether p57 is imprinted and/or mutated in WT.

In summary, 11p15 shows multiple genetic alterations in WT, including loss of a tumor suppressor gene defined by STFs in genetic complementation experiments, one or two BWS genes corresponding to balanced germline rearrangement breakpoints in BWS patients, and loss of imprinting involving *IGF2* and *H19,* outside the tumor suppressor region defined by STFs and BWS breakpoints. In addition to their individual roles in childhood cancer, it will be interesting to determine whether the physical relationship itself of these genes on 11p15 is important in the pathogenesis of Wilms' and other embryonal tumors.

ACKNOWLEDGMENTS

This work was supported by NIH grants CA54358 and CA65145.

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COMMENTARY

The existence of a second Wilms' tumor suppressor gene on chromosome 11p at band 15 (11p15) derives from the observation that many of these tumors have lost genetic material from one of the two alleles (detected by loss of heterozygosity, see manuscript of Grundy and Coppes in this issue). Feinberg provides a complementary and powerful argument for the existence of an 11p15 tumor suppressor gene. In transferring certain fragments of chromosome 11p into a particular rhabdomyosarcoma cell line, Feinberg's laboratory has been able to demonstrate that certain fragments containing 11p15 were capable of reducing the growth of the rhabdomyosarcoma cells lines. All other fragments (including those containing 11p13) did not. This strongly suggests the presence of a suppressor gene in the fragments containing chromosome 11p15. Feinberg goes on to describe the strategies used to delineate the putative Beckwith-Wiedemann gene on chromosome 11p15 (see also the manuscripts of Alders et al., and Mannens et al., in this issue of MPO) and the role of genomic imprinting of genes in this region.